Remarks

Objections to Claims

The Examiner has objected to Claim 31 because the term "SELF" is not spelled out in full at the first occurrence of that term. Applicant has amended Claim 61 to include the full term in the claim. Applicant thanks the Examiner for noting this error and respectfully requests removal of the objection.

Objection to Abstract

The Examiner objected to the Abstract as containing the legal term "means". Applicant thanks the Examiner for noting this error and has amended to remove the term "means". Applicant respectfully requests reconsideration and removal of these objections.

The § 112, First Paragraph Rejection of Claims 61-68, 70, 72, 74, and 76

The Examiner has rejected Claims 61-68, 70, 72, 74, and 76 under 35 U.S.C. § 112, first paragraph as failing to comply with the enablement requirement. The Examiner states that the claims contain subject matter that was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicant has cancelled Claim 68 and incorporated the limitations of Claim 68 into amended independent Claim 61 to claim the modification of the p53 gene using SELF non-thermal fields. Applicant respectfully traverses the rejection of amended independent Claim 61 and requests reconsideration.

The Examiner states that the breadth of the claims is too large. Applicant has amended Claim 61 to claim the modification of the p53 gene using the claimed process. Applicant respectfully submits that the instant specification contains enough information to enable one skilled in the art to practice the invention as claimed in amended Claim 61. As the Examiner notes on page 4 of the Office Action, the Applicant has demonstrated that Static and Extremely

Low Frequency (SELF) magnetic fields cause decreased gene expression in exposed mice tumors.

Further, Applicant respectfully traverses the Examiner's assertion that the prior art did not contain methods of how to use SELF non-thermal fields to modify genes before the effective filing date of the instant application. Applicant has included in Appendix I copies of two papers describing methods of modifying the expression of genes by SELF magnetic fields. Goodman, et al. describes the enhancement of the expression of RNA polymerase in *E. coli* following exposure to 72 Hz sinusoidal fields in the range of 0.07 to 1.1 mT for periods ranging from 5 minutes to 1 hour.[1] Similarly, in eukaryotic cells Lin, et al. found an increase in transcription levels for *c-myc* promoter gene after exposure to a 60 Hz sinusoidal field.[2] In Lin, et al., the field was measured at 8-80 μT (0.8-8mT). The magnetic fields described in Goodman and Lin are both well within the range of the magnetic fields claimed in amended Claim 61 of 1-30 mT as well as the frequency of 50 Hz when using AC current and an exposure time of 1-40 minutes. Applicant respectfully submits that the disclosures in these two papers, both published before the filing date of the current application, demonstrate that it was known before the filing date of the current application that SELF magnetic fields have a modifying effect on both prokaryotic and eukaryotic gene expression as measured by both increased production of RNA polymerase and transcript levels of *c-myc* promoter gene.

The Examiner states that, apart from the examples showing the SELF fields have an inhibitory effect on tumor growth, the instant specification fails to provide sufficient guidance for a skilled artisan as to how to use SELF methods on any gene modification. Applicant respectfully traverses this statement and submits that the specification provides sufficient information for a skilled artisan to use the claimed invention. Applicant notes that Claim 61 has been amended to claim modification of the p53 gene using SELF non-thermal fields and that all other claims currently under consideration depend from amended Claim 61. Further, Applicant

---

[1] Goodman, Eugene M., Ben Greenebaum, and Michael T. Marron, "Altered Protein Synthesis in a cell-Free System Exposed to a Sinusoidal Magnetic Field", *Biochimica et Biophysica Acta*, 1202, 107-122 (1993).

[2] Lin, Hana, Reba Goodman, and Ann Shirley-Henderson, "Specific Region of the *c-myc* Promoter is Responsive to Electric and Magnetic Fields, *J. Cellular Biochemistry*, 54:281-288 (1994).

respectfully submits that the operational parameters disclosed in the instant specification are similar to the operational parameters disclosed in both the Goodman and Lin papers discussed above. For example the test on confluent WiDr monolayers utilized DC and AC currents up to 100 Hz with exposure of 20 minutes. Field Intensity ranged from 0.5 to 30 mT. (See page 13, line 4 to page 14, line 2 of the specification to include Table I. These parameters are similar to the parameters described in Lin with a frequency of 60 Hz and a field intensity of 8-80 μT (0.8-8mT) with an exposure time of 5-60 minutes. Applicant notes that these parameters are also similar to those used by Goodman, et al. in modifying prokaryotic gene output as described above. Applicant respectfully submits that this similarity in operational parameters demonstrates the predictive effect of the claimed SELF process thereby enabling one skilled in the art to practice the claimed invention. Although claims must be sufficiently enabled so as not to require undue experimentation to practice the claimed invention, it is recognized that some experimentation within disclosed operational parameters by a skilled artisan may sometimes be needed to practice a claimed invention previously unknown to that skilled artisan.

Applicant also respectfully traverses the Examiner's statement that there is an absence of working examples showing how to use SELF methods of biotechnological gene modification within the range of 1 to 100mT. First, Applicant has amended Claim 61 to claim modification of the p53 gene, not biotechnological genes. Applicant has provided working examples demonstrating the effect of described SELF processes on three different cell lines. Applicant courteously notes that the Lin and Goodman papers discussed above disclose methods similar to those described in the instant specification for the working examples. Applicant respectfully submits that even if the prior art were not predictive, which it is, the described similarity of field intensity in the SELF fields in Lin and Goodman with the *in vitro* working examples in the specification provide sufficient enablement to allow a skilled artisan to use the claimed invention with only the normal experimentation always needed by a skilled artisan to practice a new method or technique.

As seen from the discussion, Applicant respectfully asserts the specification provides sufficient enablement under § 112, first paragraph to allow a person skilled in the art to practice

the claimed invention. Sufficient working examples are provided that give the operating parameters of field intensity and frequency to allow a skilled artisan to practice the invention claimed in amended Claim 61. Furthermore, Applicant has provided two papers establishing the predictability of the art prior to the filing of the instant application. Finally, Claim 61 has been amended to acclaim only the modification of the p53 gene, processes for which are described in the instant application. Applicant respectfully requests reconsideration.

Claims 62-67, 70, 72, 74, and 76 depend from amended Claim 61 and thus incorporate all the elements of that claim. Because, as discussed above, it can be seen that Claim 61 is enabled under § 112, first paragraph, it follows that claims depending from Claim 61 are also sufficiently enabled under § 112, first paragraph. Consequently, Applicant respectfully requests removal of the rejection of 62-67, 70, 72, 74, and 76 and passage to allowance of those claims.

The § 112, Second Paragraph Rejection of Claims 61-67, 70, 72, 74, and 76

Claim 61 has been amended to claim a method of using SELF non-thermal fields for modification of a p53 gene. Applicant has amended Claim 61 to eliminate the term "biotechnological" thereby rendering the rejection of Claim 61 under § 112, second paragraph moot. Applicant respectfully requests reconsideration.

Claims 62-67, 70, 72, 74, and 76 depend from amended Claim 61 and thus incorporate all the elements of that amended claim. Because the term "biotechnological" is no longer an element in Claims 62-67, 70, 72, 74, and 76, the rejection of those claims under § 112, second paragraph is also rendered moot. Applicant respectfully requests reconsideration.

The § 102 (b) Rejections of Claims 61, 63, and 67

The Examiner has rejected Claims 61, 63, and 67 as unpatentable under 35 U.S.C. § 102 (b) anticipated by United States Patent No. 5,752,911 to Canedo, et. al. ("the 911 patent"). Applicant as amended independent Claim 61 and respectfully traverses this rejection.

Applicant courteously notes that independent Claim 61 has been amended to claim a new method of using SELF non-thermal fields for modification of a specific gene, namely a p53 gene

at a field intensity of between 1 and 100mT. The '911 patent is directed only to methods of using SELF fields to treat patents suffering from epilepsy and fails to disclose the use of SELF fields on any sort of gene at all, let alone the specific p53 gene. Moreover, the '911 patent further limits the use of SELF fields on patents who suffer epileptic seizures while under medication. (See col. 4, lines 43-46 of the '911 patent.) Consequently, there is no disclosure of the effect of SELF fields on normal or control persons and consequently no disclosure of the effect of SELF fields on normal p53 genes. In contrast, Table 2 (page 15) of the instant specification and the accompanying text (pages 15, line 4 through page 16, line 2 of the specification) disclose the differences in the effect of SELF fields on the p53 gene of malignant cells versus the effect of the same fields on the p53 gene of normal cells.

In summary, with the instant application, Applicant has disclosed and claimed in amended Claim 61 a new method of use of SELF non-thermal fields not disclosed in the '911 patent. For this reason, Applicant respectfully requests withdrawal of the rejection of amended independent Claim 61 under § 102 (b) and passage to allowance of that claim.

Claims 63 and 67 depend from Claim 61 and thus incorporate all the element of that claim. Therefore, because the method of using SELF non-thermal fields to modify a p53 gene claimed in amended Claim 61 is not anticipated by the '911 patent, dependent Claims 63 and 65 are also not anticipated by the '911 patent. Applicant respectfully requests reconsideration ad passage to allowance of Claims 63 and 65.

Conclusion

Applicant respectfully submits that the present application is now in condition for allowance, which action is courteously requested. The Examiner is invited and encouraged to contact the undersigned attorney of record if such contact will facilitate an efficient examination and allowance of the application.

Respectfully submitted,

C. Richard Lohrman
Registration No. 46,878
ATTORNEY FOR APPLICANT
Simpson & Simpson PLLC
Customer No. 24041
5555 Main Street
Williamsville, NY 14221
Phone: (716) 626-1564
Fax: (716) 626-0366

Dated: July 12, 2004

APPENDIX I

Journal of Cellular Biochemistry 54:281–288 (1994)

Specific Region of the c-*myc* Promoter Is Responsive to Electric and Magnetic Fields

Hana Lin, Reba Goodman, and Ann Shirley-Henderson

Department of Pathology, Columbia University Health Sciences, New York, New York 10032 (H.L., R.G.) and Institute for Biomolecular Structure and Function (A.S.-H.), Biological Sciences, Hunter College-CUNY, The Graduate Division of the City University of New York, New York 10021

Abstract The level of c-*myc* transcripts is increased in cells exposed to extremely low frequency (elf) electromagnetic (EM) fields at 60 Hz. The aim of the present experiments was to determine if regulatory regions upstream of the c-*myc* gene modulate the response to EM fields. DNA upstream of P1 of both mouse and human c-*myc* genes was transfected into cells as CAT constructs. The presence of DNA 5′ to the human or mouse myc genes results in increased expression of CAT following 20 min exposures of cells to 60 Hz elf EM fields. Specific portions of the human upstream DNA were deleted and introduced into cells. The region responsive to EM fields is located between −353 and −1,257 relative to the P_1 promoter.

Key words: c-*myc* promoter, CAT expression, low frequency electric and EM fields, transcription

The possibility of health risks resulting from exposure to electric and magnetic (EM) fields provides a strong motivation to determine how such fields interact with cells. Initially, an important clue to understanding how cells respond to EM fields was the finding that transient increases in specific transcripts occur in cells exposed to extremely low frequency [(elf) (>300 Hz)] EM fields. This response has been observed in a variety of cell types including dipteran salivary gland cells [Goodman et al., 1983, 1992a,b, in press], yeast cells [Weisbrot et al., 1993a], and human cells [Goodman and Henderson, 1991; Goodman et al., 1992c,d; Czerska et al., 1992; Phillips et al., 1992; Liburdy et al., 1992]. The effect of EM fields is probably directly at the transcriptional level [Goodman et al., 1983; Phillips et al., 1992]. The initial evidence came from analysis of transcription autoradiograms of dipteran salivary gland cells. The presence of increased grain density over specific chromosome regions indicated a direct influence of low frequency EM fields on transcription per se rather than, for example, an increase in RNA stability, or the release of RNA storage forms [Goodman et al., 1983, 1992a,b, in press]. Other experimental evidence for effects at the transcription level is derived from nuclear run-off analyses. Increased transcription of c-*myc*, c-*fos*, c-*jun*, and protein kinase C was observed in a derivative of human T-lymphoblastoid cells [Phillips et al., 1992].

Transcript levels for c-*myc* are increased in a variety of cell types exposed to a 60 Hz sinusoidal EM field, and under different experimental conditions. Increased c-*myc* transcript levels have been measured following short exposures of thymocytes stimulated with Con A to EM fields [Liburdy et al., 1992]. The increase in transcript levels is coordinate with an increase in intracellular calcium, which implies an interaction of EM fields with the cell membrane. The increase in transcripts following a 60 Hz EM field exposure is rapid, within 4–8 min [Goodman et al., 1992c]. Taken together, these results strongly suggest that regulatory pathways are implicated in the response of the cell to low energy EM fields.

DNA upstream of c-*myc* was transfected into both mouse (stable transfectants) and human cells (transient transfectants) as a CAT construct. The presence of upstream DNA in CAT constructs resulted in increased expression of

Abbreviations: CAT, chloramphenicol transferase; elf, extremely low frequency; EM, electric and magnetic; Hz, Hertz.

Received September 8, 1993; accepted October 25, 1993.

Address reprint requests to R. Goodman, Columbia University Health Sciences, Department of Pathology, 630 W. 168th Street, New York, NY 10032.

CAT after exposure of cells to 60 Hz EM fields. Further experiments showed that the upstream regulatory region responsive to EM fields in human cells is approximately 900 base pair (bp) and located between the restriction sites ClaI and PvuII.

MATERIALS AND METHODS

Cell Culture and Transfections

HeLa cells are maintained in D-MEM (Gibco), 10% fetal calf serum (FCS). Mouse myeloma (PX3) cells are grown in IMDM (Gibco). All media included 10% FCS and 1% Penn-Strept.

Transfections of cells used lipofectin (HeLa cells) and electroporation (mouse cells). Mouse cells were stably transfected [Muller et al., 1990]. For electroporation, the cells were seeded at 5×10^5 per 25 cm plate and incubated overnight in medium. After washing, the cells were suspended at 5×10^5 cells per ml in cold PBS. Twenty micrograms of linearized pSV2-neo DNA (linearized with PstI) was added to 1 ml of cells, and the cells placed on ice for 15 min. Electroporation used 220 volts, 960 capacitance. The cells were returned to ice for 30 min after electroporation. They were washed in 10% FCS IMDM and subcloned by placing the cells in a 96 well plate for 24 hr in selective media. After 24 hr, the media was replaced. The cells were maintained for about 4 weeks in 0.5 mg/ml G418. Eight resistant clones were pooled and maintained as cell lines.

For transient transfections, HeLa cells were placed in 10% FCS EMDM for 3 hr before transfection [Malone et al., 1989; Zhang et al., 1993]. Ten micrograms of DNA was mixed well in 100 μl of OPTI-MEMI (serum free). Other procedures are as described with the Gibco lipofectin transfection kit. From the final mixture 2.75 ml was added to a culture dish containing 8×10^5 cells/ml in a T-25 dish. The mixture was incubated overnight or 15 hr. After 15 hr, the media was replaced with normal media. The cells were harvested for CAT assay 48–63 hr after transfection.

Plasmid Construction

Murine c-*myc* DNA (1.8 kilobase) (gift of Dr. K. Calame, Columbia University) was subcloned into PUC-18 containing CAT by blunt end ligation. CAT was originally excised from pSV2-CAT using a HindIII-EcoRI digest. Portions of the human myc upstream regulatory promoter regions were obtained from Dr. R. Dalla-Favera. These included the following regions: (1) a 2.8 kb HindIII to PvuII digest (HPV); (2) a 1.7 kB ClaI to PvuII fragment (CPV); and (3) a PvuII to PvuII fragment of 0.86 kb. (PPV) Blunt end ligation into pSV_2 CAT used a HindIII linker.

CAT Assay

CAT was measured by enzymatic determinations. Acetylated ^{14}C-chloramphenicol was measured by thin layer chromatography [Gorman et al., 1982]. Assays contained 30–60 μg of extract protein as measured by the BioRad Protein assay kit. After chromatography, spots were quantitated using a beta scanner, or by scintillation counting. CAT activity was calculated as the percentage of chloramphenicol converted to the acetylated form.

RNA Extraction and Analysis

RNA isolation and purification has been described [Goodman et al., 1992c].

Quantitation of Transcript Levels

For dot blot analysis [Muller et al., 1982], 4 μg of total RNA was used from each sample for dot blot and diluted by half for each point. Each sample was probed for c-*myc* and CAT. CAT DNA was labeled in vitro with ^{32}P-dCTP using the random primer method [Weinberg and Vogelstein, 1983] (Amersham Random Primer Kit). Hybridization was performed at 45°C for 12 hr and the membrane was washed at 65°C (30 min) twice, with 2× SSC, 1% SDS, 0.1 SSC, and 1% SDS. The membrane was exposed overnight at −70°C. Dot blot quantitation was obtained by measuring the radioactivity of each dot using a Packard Tri-Carb 4530 scintillation counter. The sensitivity of the dot blot process was measured by dotting equal amounts of control RNA onto a filter and quantitating the radioactive counts. The standard error is about 5%. Two randomly chosen areas of each filter were measured for background. Proportionality was observed between experimental and control dots at the various concentrations. All samples were examined for DNA contamination, and monitored for RNA breakdown, using agarose gel electrophoresis before hybridization studies.

Northern Hybridization

For Northern hybridization, 15 μg of total RNA was used for each sample; 50 ng of CAT DNA was labeled as a probe using the random

primer method. Hybridization was at 45°C for 12 hr and membrane was washed at 65°C (30 min) twice, with 2× SSC, 1% SDS, 0.1 SSC, and 1% SDS. Membrane was exposed overnight at −70°C. Probe DNA was isolated by agarose gel electroelution and subsequently purified (Geneclean, La Jolla, CA). DNA probes were labeled in vitro with [^{32}P]dCTP via the random primer method.

Composition of EM Signals

All exposures used a continuous sinusoidal 60 Hz field generated by a pair of Helmholtz coils (Electro-Biology Inc., Parsippany, NJ) [Goodman et al., 1992c]. EM fields used were 8 or 80 μTelsa. The calculated corresponding induced electric fields were 50 and 500 μV/meter [Bassen et al., 1992]. Exposures were for up to 20 min in duration.

Conditions of EM Field Exposure

HeLa or mouse myeloma (PX3) cells were changed to new media 3 hr before exposure in 100 × 20 mm culture dishes. PX3 cells from a single flask were divided into two flasks to be used as experimental and control cells (1×10^6 cells/ml). Cells from three flasks of transfected HeLa cells were combined into two flasks for exposures. One flask was placed in the exposure apparatus while the remaining flask served as a simultaneous control. Details on the placement of cells, physical separation of experimental and control flasks, and composition and construction of the mu metal container shielding the exposure signal are described in Goodman et al. [1992c]. Control cells were placed in a mu metal box in the same incubator as experimental cells to minimize potential thermal differences that could arise by using separate incubators. The signal generator was placed outside the incubator. All experiments were carried out at 37°C. Temperature was monitored using a Physitemp thermocouple temperature probe (Physitemp Instruments Inc., Clifton, NJ) which is sensitive to 0.1°C.

RESULTS

Construction of Transfectants in Mouse Myeloma Cells; DNA Upstream of the *myc* Promoter Increases the Expression of CAT in Myeloma Cells Exposed to a 60 Hz EM Field

Stable transfections of mouse myeloma cells (PX3) used a CAT construct containing 1.8 kb of the murine promoter including exon 1 and flanking sequences to −1,141 relative to P_1 (Fig. 1). The relative quantity of the c-*myc* transcript was the same in both transfected and nontransfected cells. Parental and transfected mouse myeloma cells were exposed to a 60 Hz sinusoidal field to test the response relative to time of exposure for CAT expression (Fig. 2A–D).

The maximum effect on either c-*myc* or CAT transcript increase in murine myeloma cells was at an EM field of 80 μT and 20 min of exposure. CAT enzymatic activity was also increased in cells exposed to 60 Hz EM fields under the same conditions (Fig. 3A,B). The results clearly indicate that DNA upstream of the c-*myc* promoter is responsive to the EM field in stably transfected cells.

Transient Assays Using HeLa Cells; DNA Upstream to the *myc* Gene Increases the Expression of CAT in Human Cells Exposed to a 60 Hz Sinusoidal EM Field

Transiently transfected HeLa cells were exposed to a 60 Hz sinusoidal field at both 8 and 80 μT. The maximum exposure conditions were at 8 μT for 20 min (Fig. 4). Three chimeric con-

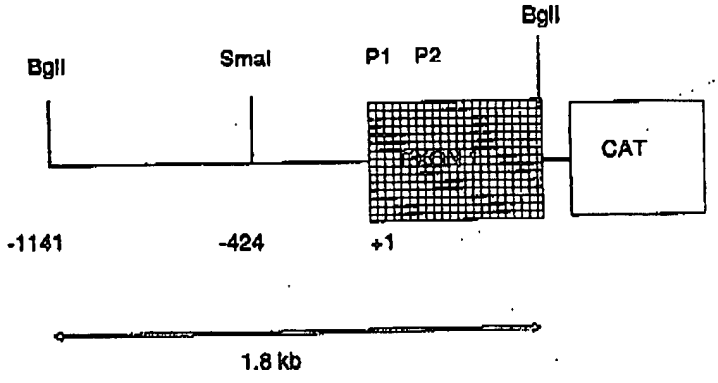

Fig. 1. Diagram of murine c-*myc* upstream regulatory region CAT construct.

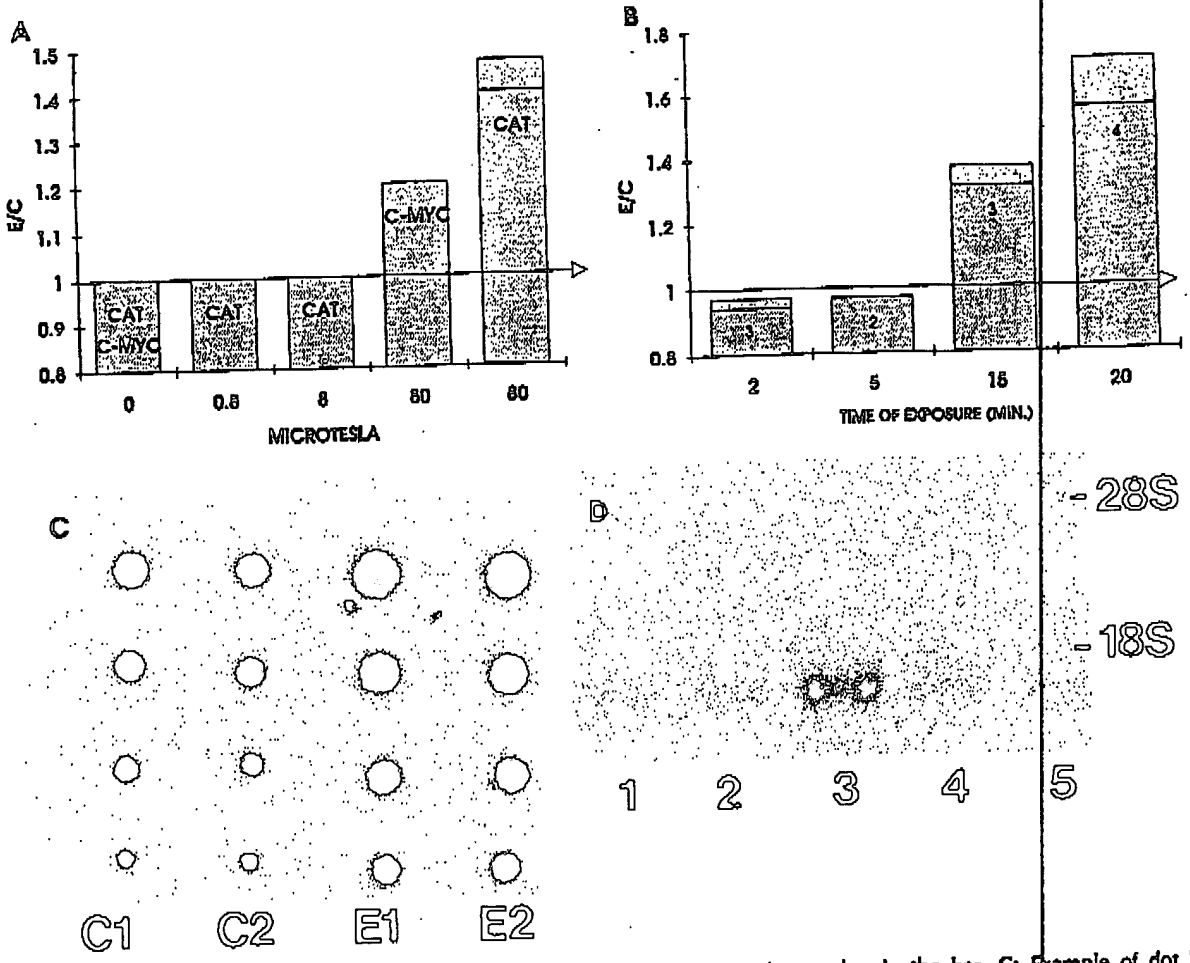

Fig. 2. Determining exposure conditions for maximum transcript levels in stably transfected mouse cells. The results in A and B are expressed as the ratio of experimental to control (E/C). The dark portion of each bar represents the standard error of the mean where three or more experiments were performed. A: The effect of increasing field strength on transcript levels for CAT and c-*myc*. The maximum effect was at 80 μT, 100 μV/m, and 20 min of exposure. Values were determined by dot blot hybridizations using total RNA against c-*myc* or CAT DNA. Control values for myc and CAT are the mean of three experiments; the 0.8 and 8 μT points are the mean of two experiments. The 80 μT points for myc are the mean of two experiments; for CAT, the mean of 10 experiments. B: The effect of increasing time of exposure on transcript levels for CAT and c-*myc* transcripts. The number of experiments represented is given by the number in the bar. C: Example of dot blot hybridization of total murine RNA against CAT DNA. CAT expression was measured between unexposed control cells and exposed cells (80 μT for 20 min). CAT DNA was labeled in vitro with ^{32}P-dCTP using random primer. Hybridization conditions were as described in A. Control cells, C; exposed cells, E. D: Northern blot hybridization against CAT DNA. Northern blot hybridization for CAT gene expression was measured in cells exposed to each of three field strengths: 0.8 (lane 1), 8 (lane 2), and 80 μT (lane 3). Lanes 4 and 5 are RNA from unexposed cells. Hybridization was performed at 45°C for 12 hr and the membrane was washed at 65°C (30 min) twice, with 2× SSC, 1% SDS, 0.1× SSC, and 1% SDS. The membrane was exposed overnight at −70°C.

structs containing portions of the c-*myc* promoter upstream of CAT were used in transient assays (Fig. 5). CAT expression for each construct is given in Table I. The HPV and CPV constructs caused increased expression in the presence of the EM field, but the levels of expression using the PPV construct were the same under control and exposure conditions (Fig. 6). Maximal activity was achieved with a chimera constructed from −2,329 (from P_1) to the second PvuII site in exon 1 (HPV) of the c-*myc* gene. Transfection of the constructs containing 1,257 bp of upstream DNA (CPV) showed about 70% of the expected activity; the construct containing the −353 bp (PPV) fragment gave about 50% of the maximum activity. Based on previous reports [Hay et al., 1987], it was expected that the value for the first 353 bp of upstream sequence would be about 10% of the maximum. We were unable to resolve this discrepancy, even

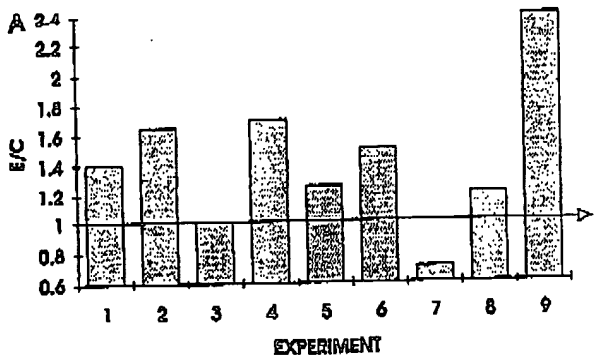

Fig. 3. Determination of CAT activity on murine transfected cells in presence and absence of an EM field. Mouse c-*myc* upstream DNA (1.8 kb) in a CAT construct was transfected into mouse myeloma (px3) cells. Acetylated ^{14}C-chloramphenicol was separated by thin layer chromatography. A: The results of a series of experiments where a stable clone of transfected mouse cells was exposed to an 80 μT, 100 μV/m elf EM field for 20 min. The mean ratio of the exposed to control cells for the series was 1.44 ± 0.16 (SE mean). Experiments 1–6 used direct counting of gel; 7–9 used beta scanner for determinations of radioactivity. B: Example of results of thin layer chromatography.

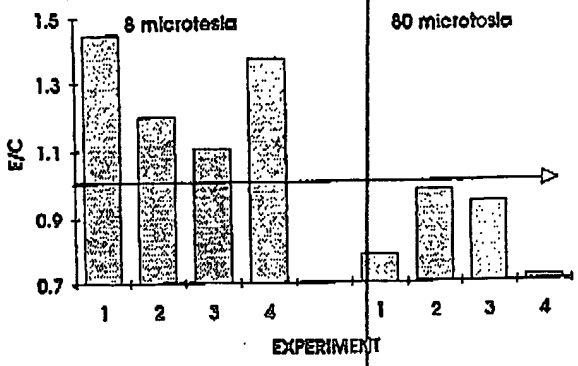

Fig. 4. Determination of conditions for EM field exposure in HeLa cells; the effect of increasing field strength on transcript level for CAT. Transfection of the entire 2.3 kb of DNA upstream to the c-*myc* gene was used in these experiments. Exposure of transfected HeLa cells to EM fields was at 8 and 80μT for 20 min. Values were determined by dot blot hybridizations. The mean of the four different experiments (using four separate transfections) for exposure at 8 μT was 1.2 ± 0.07 (SE mean); at 80 μT, it was 0.9 ± 0.06 (SE mean). A significant increase was seen with exposure at 8 μT and 100 μV/m at 20 min of exposure.

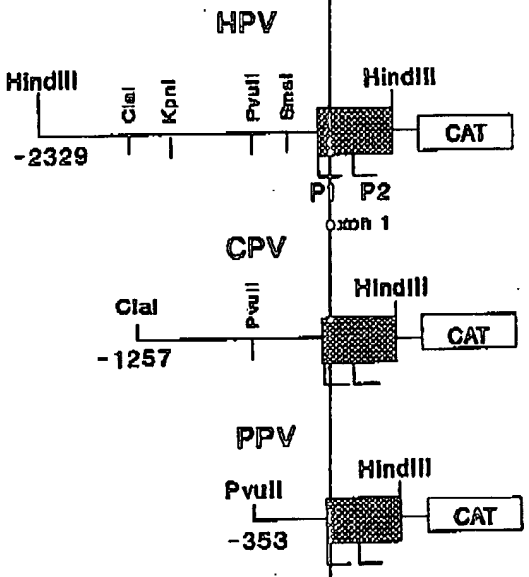

Fig. 5. Schematic diagram of the three constructs of human DNA upstream of the c-*myc* gene with CAT.

using a series of dilutions for the CAT assay, and assume that the differences in cell types used in the transfections may be the cause.

DISCUSSION

A targeted interaction between EM fields and the cell membrane has been proposed as one route by which a cell could respond to EM fields [Adey et al., 1982; Blackman et al., 1989; Wallaczek and Liburdy, 1990]. It is assumed that a change in surface charge influences receptor binding activity either directly or indirectly through changes in the calcium flux patterns of the cell. Identification of both frequency and intensity windows support this idea [Goodman and Henderson, 1991; Blackman et al., 1989; Wei et al., 1990].

The nature of the subset(s) of genes that respond to EM fields has yet to be identified, but exposure of cells to low frequency EM fields does

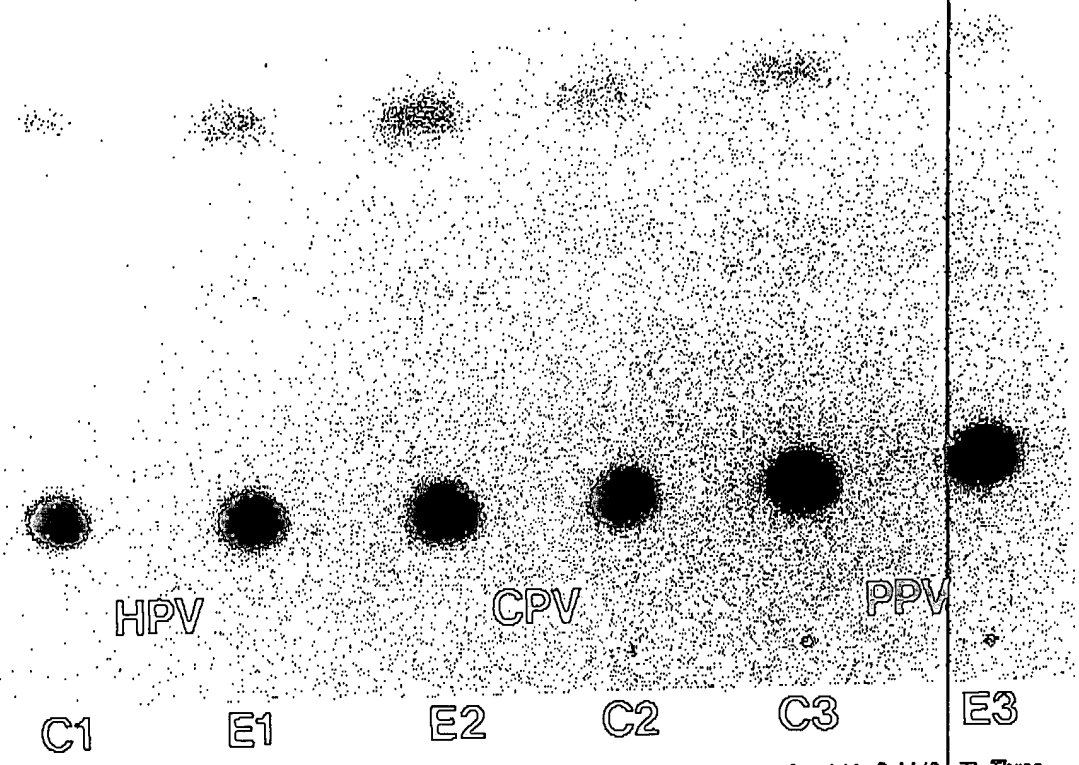

Fig. 6. Determination of CAT activity in transfected cells in the presence and absence of a 60 Hz field (8 μT). Three constructs were transfected into HeLa cells. The HPV and CPV constructs caused increased CAT gene expression in the presence of the EM field. Transfected cells using each construct were divided into two dishes for control and exposed conditions. The levels of expression using the PPV construct were the same under control and exposure conditions (see Fig. 5 and Table I). C, control; E, exposed.

TABLE I. Regulation by Upstream DNA Sequences in c-*myc* Promoter in the Presence and Absence of an EM Field

	HPV		CPV		PPV	
	C	E	C	E	C	E
# Determinations[a]	13	14	5	5	5	5
Mean CAT activity	0.31	0.59	0.22	0.38	0.16	0.16
SE mean	0.03	0.06	0.04	0.085	0.02	0.02
Activity (relative to HPV)	1	1.9	0.72	1.2	0.5	0.5
Ratio of activity of exposed and control cells		1.9		1.7		1

[a]Each determination used a separate transfection (see *Materials and Methods*).
Upstream DNA regions are defined in Figure 6. Exposed cells were placed in a B field of 8 μT for 20 min. Relative CAT activity was determined by chromatographic assay. Radioactivity was determined by beta scanning.

not affect all expressed genes [Phillips et al., 1992; Blank et al., 1992]. An increase in the myc transcript has been measured by several investigators in cells exposed to various electric and/or EM fields. The types of cells affected include derivatives of a human T-lymphoblastoid line [Phillips et al., 1992], Con-A–stimulated rat thymocytes [Liburdy et al., 1992], Daudi cells [Czerska et al., 1991], HL-60 cells [Goodman and Henderson, 1991; Goodman et al., 1992c,d; Weisbrot, 1993b; Blank et al., 1992], SV-40 transformed human fibroblasts [Gold et al., in press], and, in the present study, both HeLa and mouse myeloma cells. These findings are important to determining a mechanistic pattern for the effects of EM fields on cells since regulation of the c-*myc* gene plays an important role in initiation and continuance of normal cell proliferation, as well as in the inception of cancer.

c-*myc* expression is regulated by many factors that include transcriptional initiation and elongation, stability of the mRNA [Levine et al.,

1986; Bentley and Groudine, 1986; Hay et al., 1987, 1989], and downregulation of c-*myc* by retention of pol II at the transcription start site [Strobl and Eick, 1992]. The human gene contains three exons that encode the major product of c-*myc* [Hann and Eisenman, 1984]. Transcription can begin at either one of three sites that are regulated by different promoters, designated P_0, P_1, and P_2 [Battey et al., 1983]. P_0 is a minor promoter, accounting for less than 10% of the total transcription from the c-*myc* gene [Bentley and Groudine, 1986]. The majority transcript is controlled by the P_2 promoter. The P_1 and P_2 promoters respond to many of the same positive and negative regulators located upstream of the gene [Hay et al., 1987]. Hay et al. [1987, 1989] identified a region between −293 and +513 (relative to P_1) that is sufficient for activity of P_1 and P_2. There are two additional regions that exert positive effects on P_1 and P_2 from −353 to −1,257 and −1,257 to −2,329. A negative regulator for both promoters is located at −293 to −353. It contains two or more regulatory regions with binding properties consistent with an AP-1 site and an overlapping octomer site [Hay et al., 1987, 1989]. The positive regulators upstream of the negative regulator region can, at least in part, negate the effect of the negative regulatory element (NRE).

The present results show that at least one effect of EM on cells involves regulation of transcription. The presence of DNA upstream to myc resulted in increased expression of CAT following exposure of cells to a 60 Hz sinusoidal field. An increase in expression was observed when the HPV (−2,359 to +513) and CPV (−1,257 to +513) constructs were present in HeLa cells. There was no difference, however, in the values obtained from exposed and control cells when only the PPV (−353 to +513) construct was present. One conclusion is that a critical sequence responsive to the 60 Hz field lies within the approximately 900 bp region difference between the CPV and PPV constructs. An alternate conclusion, however, is that the negative regulatory element within the PPV construct, in the absence of upstream positive regulators, can override the effect of EM fields.

The function of the myc protein as a transcription factor must involve a myriad of genes, considering the proposed roles for c-*myc* in the cell. An affect of EM fields on this type of regulatory gene is important in light of the diversity of effects in cells and organisms that have been ascribed to EM field exposure. Possible effects on regulatory genes could provide a plausible means of defining altered activity in cells resulting from EM field exposure, as well as a putative role for the involvement of signal transduction pathways. From a biological point of view, this mechanistic approach is reasonable since it is consistent with the observed transcriptional activation, and other biological effects attributed to EM field exposure. Proof of mechanism, however, will require demonstration of a feasible means of interaction at the cell membrane and determination of the pathway(s) from cell surface to the DNA in the nucleus.

ACKNOWLEDGMENTS

We thank Dr. B. Tycko of the Division of Pediatric Oncology in the Department of Pathology, Columbia University Health Sciences, for reading this manuscript critically. Portions of this work were supported by the Office of Naval Research [NOOO14-90-J-0577 (R.G.) and NOOO14-90-J-1266 (A.H.)], DOE Project 19X-SK733C, and the NIEHS 1RO1 ES(CA)-05735-01. Part of this research was performed under the auspices of the RCMI Center for Gene Structure and Function at Hunter College (Institute for Biomolecular Structure and Function).

REFERENCES

Adey WR, Bawin SM, Lawrence AF (1982): Effects of weak amplitude-modulated microwave fields on calcium efflux from awake cat cerebral cortex. Bioelectromagnetics 3:295–308.

Bassen H, Litovitz T, Mister R (1992): ELF in vitro exposure systems for inducing uniform electric and magnetic fields in cell culture. Bioelectromagnetics 13:183–198.

Battey J, Moulding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G, Leder P (1983): The human cell oncogene: Structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell 34:779–787.

Bentley DL, Groudine M (1986): A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL-60 cells. Nature 321:702–706.

Blackman CF, Kinney LS, House DE, Joines WT (1989): Multiple power-density windows and their possible origin. Bioelectromagnetics 10:115–128.

Blank M, Soo L, Lin H, Henderson AS, Goodman R (1992): Changes in transcription in HL-60 cells following exposure to alternating current from electric fields. Bioelectrochem Bioenerg 28:301–309.

Czerska E, Al-Barazi H, Casamento J, Davis C, Elson E, Ning J, Swicord M (1991): Comparison of the effect of elf fields on oncogene expression in normal and transformed human cells. In: "Transactions of the Bioelectromagnetic Society, 13th Annual Meeting." Frederick, MD: W/L Associates Ltd., pp B2–B14.

Gold S, Goodman R, Henderson A (in press): Exposure of human cells to electric and magnetic fields results in increased levels of T-antigen mRNA and protein. Bioelectromagnetics.

Goodman R, Henderson AS (1991): Transcription in cells exposed to extremely low frequency electromagnetic fields: A review. Bioelectrochem Bioenerg 25:335–355.

Goodman R, Bassett CAL, Henderson AS (1983): Pulsing electromagnetic fields induce cellular transcription. Science 220:1283–1285.

Goodman R, Weisbrot D, Uluc A, Henderson A (1992a): Transcription in *Drosophila* melanogaster salivary gland cells is altered following exposure to low frequency electromagnetic fields: Analysis of chromosome 3R. Bioelectromagnetics 13:111–118.

Goodman R, Weisbrot D, Uluc A, Henderson A (1992b): Transcription in *Drosophila* melanogaster salivary gland cells is altered following exposure to low frequency electromagnetic fields: Analysis of chromosomes 3L and X. Bioelectromagnetics 28:311–318.

Goodman R, Wei L-X, Bumann J, Henderson A (1992c): Exposure to electric and magnetic (EM) fields increases transcripts in HL-60 cells: Does adaptation to EM fields occur? Bioelectrochem Bioenerg 29:185–192.

Goodman R, Wei L-X, Bumann J, Henderson AS (1992d): Exposure of human cells to electromagnetic fields: Effect of time and field strength on transcript levels. Electro Magnetobiol 11:19–28.

Gorman CM, Moffat LF, Howard BH (1982): Recombinant genomes which express chloramphenicol acetyl-transferase in mammalian cells. Mol Cell Biol 2:1044–1051.

Hann SR, Eisenman RN (1984): Proteins encoded by the human oncogene: Differential expression in neoplastic cells. Mol Cell Biol 4:2486–2497.

Hay N, Bishop JM, Levens D (1987): Regulatory elements that modulate transcription from human c-myc. Genes Dev 1:659–671.

Hay N, Takimoto M, Bishop JM (1989): A FOS protein is present in a complex that binds a negative regulator of MYC. Genes Dev 3:293–303.

Levine RA, McCormick JE, Buckler A, Sonenshein GE (1986): Transcriptional and posttranscriptional control of c-myc gene expression in WEHI 231 cells. Mol Cell Biol 6:4112–4116.

Liburdy R, Callahan DE, Harland J, Sloma T, Yaswen P (1992): Signal transduction in the lymphocyte is influenced by 60 Hz magnetic fields: Mitogen binding, calcium and mRNA induction. San Diego: The Annual Review of Research on Biological Effects of Electric and Magnetic Fields from the Generation, Delivery and Use of Electricity, Poster 12.

Malone RW, Felgner PL, Verma IM (1989): Cationic liposome-mediated RNA transfection. Proc Natl Acad Sci USA 86:6077–6081.

Muller R, Slamon D, Tremblay J, Cline M, Verma I (1982): Differential expression of cellular oncogenes during pre- and postnatal development of the mouse. Nature (London) 299:640–644.

Muller SR, Sullivan PD, Clegg DO, Feinstein SC (1990): Efficient transfection and expression of heterologous genes in PC12 cells. DNA Cell Biol 9:221–229.

Phillips JL, Haggren W, Thomas W, Ishida-Jones T, Adey WR (1992): Magnetic field induced changes in specific gene transcription. Biochim Biophys Acta 1132:140–144.

Strobl LJ, Eick D (1992): Hold back of RNA polymerase II at the transcriptional start site mediates down-regulation of c-myc in vivo. EMBO J 11:3307–3314.

Wallaczek J, Liburdy R (1990): Non-thermal 60 Hz sinusoidal magnetic-field exposure enhances $^{45}Ca^{+2}$ uptake in rat thymocytes. Dependence on mitogen activation. FEBS Lett 271:157–160.

Wei L-X, Goodman R, Henderson AS (1990): Changes in levels of c-myc and histone H2B following exposure of cells to low frequency sinusoidal signals: Evidence for window effect. Bioelectromagnetics 11:269–272.

Weinberg A, Vogelstein B (1983): A technique for radiolabelling DNA restriction endonuclease fragments for high specific activity. Anal Biochem 132:6–13.

Weisbrot DR, Khorkova O, Lin H, Henderson A, Goodman R (1993a): The effect of low frequency electric and magnetic fields on gene expression in Saccharomyces cerevisiae. Bioelectrochem Bioenerg 31:167–177.

Weisbrot D, Uluc A, Henderson A, Goodman R (1993b): Transcription in *Drosophila* melanogaster salivary gland cells is altered following exposure to low frequency electric and magnetic fields: Analysis of chromosomes 2R and 2L. Bioelectrochem Bioenerg 31:39–47.

Zhang Y, Shields T, Crenshaw T, Hao Y, Moulton T, Tycko B (1993): Imprinting of human H19: Allele-specific CpG methylation, loss of active allele in Wilms tumor, and potential for somatic allele switching. Am J Hum Genet 53:113–124.

Biochimica et Biophysica Acta, 1202 (1993) 107–112

BBAPRO 34554

Altered protein synthesis in a cell-free system exposed to a sinusoidal magnetic field

Eugene M. Goodman [a], Ben Greenebaum [a] and Michael T. Marron [b]

[a] *Biomedical Research Institute, University of Wisconsin-Parkside, Kenosha, WI (USA) and*
[b] *Office of Naval Research, Arlington, VA (USA)*

(Received 10 November 1992)
(Revised manuscript received 23 March 1993)

Key words: Cell-free expression system; Transcription/translation; Magnetic field; RNA polymerase

This report describes a new approach for examining weak extremely low frequency (ELF) electric and magnetic field interactions with living systems that exploits a cell-free transcription/translation system derived from *Escherichia coli*. Using two-dimensional polyacrylamide gel electrophoresis we previously had determined that the level of the α subunit of RNA polymerase in intact *E. coli* was elevated by exposure to weak ELF magnetic fields. In this paper, plasmids containing the α, or both the β,β' subunits of the RNA polymerase from *E. coli* were placed into a cell-free expression system. When this transcription/translation system was exposed to a 72-Hz sinusoidal magnetic field in the range 0.07 to 1.1 mT (rms) for periods of 5 min to 1 h, expression was enhanced. Weaker fields must be applied longer to produce an effect. For 10 min of field exposure, the threshold for an effect is 0.1 mT. These experiments demonstrate that an intact membrane is not an absolute requirement for transducing magnetic bio-effects.

Introduction

In recent years both the public and the scientific community have expressed concern about the potential hazards associated with exposure to weak extremely low frequency (ELF) electric and magnetic fields. One basis for this concern are epidemiological data suggesting that field exposure results in an increased incidence of certain cancers [1–4]. An important factor fueling the contentious nature of these discussions has been the absence of a plausible mechanism explaining how weak fields interact with biological tissue to induce the reported bio-effects. Most proposed mechanistic theories suggest that the plasma membrane is either directly or indirectly involved in transducing an ELF field perturbation [5,6]. These membrane-based theories are plausible in terms of the electric field component of an applied field, but generally ignore any direct role for a magnetic field component.

In an earlier study using two-dimensional polyacrylamide gel electrophoresis with intact *Escherichia coli*, the level of the α subunit of RNA polymerase was found to be elevated following magnetic field (MF) exposure [7]. RNA polymerase is comprised of four subunits and is responsible for RNA transcription in *E. coli*. The four subunits (α_2 and β,β') together with a sigma factor, are all required for in-vivo transcription. We have employed two plasmids in these experiments, one encoding the α subunit and one encoding the β,β' subunits. In many of the experiments a mixture of roughly equivalent amounts of each plasmid was used.

This paper describes experiments using a cell-free expression system that demonstrate enhanced expression following exposure to 72-Hz sinusoidal fields in the range 0.07 to 1.1 mT (rms) for periods of 5 min to 1 h. Weaker fields must be applied longer to produce an effect. Although an intact membrane was not present during exposure to MFs, further study is needed to determine the role, if any, of protein-lipid interactions.

Materials and Methods

The cell-free expression system [8] was prepared using *E. coli* BL-21 obtained from Dr. Richard Burgess, McArdle Laboratory for Cancer Research (Madison, WI, USA). 10 ml of an overnight BL-21 culture were transferred to 2 liters of new LB medium and grown to late log phase ($OD_{600\,nm} = 0.7$) before cells were isolated and washed twice in buffer. Bacteria were broken

Correspondence to: E.M. Goodman, Biomedical Research Institute, University of Wisconsin-Parkside, Kenosha, WI 53141, USA.

in a French pressure cell (Aminco™) at 2400 psi and 4°C; the supernatant was centrifuged twice at 30 000 rpm in a Beckman SW 28.1 rotor at 4°C. The supernatant was extensively dialyzed and later optimized for Mg^{2+}, Ca^{2+} and NH_4^+ ions using a plasmid with a β-galactosidase reporter gene. The optimization for each batch of extract was accomplished by experimentally determining the concentration of each cation that gave maximal expression of β-galactosidase [9]. The cell-free extracts (referred to here as S-30) were stored in 180-μl aliquots at −70°C.

The reaction mix used for experiments was prepared in a single tube as described by Zubay [8] and modified by Jovanovich et al. [9]. The mix contained various salts, nucleotides (40 mM ATP, 10 mM CTP, 10 mM UTP, 10 mM GTP), 1 mM cAMP, 20 μg/ml folinic acid, 1 mM dithiothreitol, 150 μl of the S-30 fraction, 19 amino acids (all at 0.5 mM, no methionine) and 0.5 μl of [^{35}S]methionine (spec. act. 47.5 TBq/mM). In all experiments, the previously described reaction mix was combined with the cell-free, S-30 extract in a 20:15 ratio.

After the components were mixed, they were redistributed in 48-μl aliquots into 10 polystyrene tubes (10 × 75 mm) and approx. 0.5 μg of a DNA plasmid was added to 8 of the tubes. Four of the eight tubes containing plasmid were then randomly selected as controls (no applied fields) and four as experimentals that would be exposed to MFs. The two remaining tubes with all reactants except the plasmid were used as negative controls: one was run simultaneously with the control and the other with the MF-exposed system. Plasmids (pNO2530 and pNO2661) containing, respectively, the α and β,β' subunits of RNA polymerase, originally constructed by Bedwell and Nomura [10], were obtained from Dr. Richard Burgess, McArdle Laboratory for Cancer Research. Because expression of the α and β,β' subunits was controlled by the *LacZ* promotor, enough of the inducer isopropyl-β-D-thiogalactopyranoside was added to the reaction mix so that its final concentration would be 2 mM. Plasmids used in these experiments were purified by CsCl centrifugation.

In the first set of experiments, cell-free reaction mixtures containing the α plasmid, β,β' plasmid, or a mixture of the two were exposed to 72 Hz fields of 1.1 mT for 60 min. Similarly exposed samples containing the α plasmid were also used for Western blots to analyze the protein expressed in the cell-free system. A second set of experiments using a 50:50 mixture of the two plasmids examined the role of time and intensity of exposure on expression. Fields of 0.07, 0.21 and 1.1 mT were applied for periods of time ranging from 5 to 45 min.

After exposure, the tubes were removed from the control and exposure incubators and 5 μl of a 10 mM non-labelled methionine chase solution was added to complete already initiated translation. Following a 10 min chase, all translation was stopped by adding 0.5 ml of 1 M NaOH and incubating the mixture at 37°C for 15 min; 3.0 ml of 25% trichloroacetic acid (TCA) was then added to precipitate radiolabelled protein. The contents of the entire tube were filtered and the precipitate collected on 25 mm (0.45 μ) cellulose nitrate filters (MFS, Dublin, CA, USA) supported on a Hoefer 10-place manifold. The precipitate was washed three times in cold 5% TCA, followed by a wash in 95% ethanol. The filters were air-dried, placed in 20 ml of ReadySafe™ cocktail (Beckman) and counted in a Tracor scintillation counter. To perform a Western blot, 2.5-μl samples were removed directly from the control and exposed cell-free reaction mixtures, placed in 200 μl of lysis buffer and boiled for 3 min [11]. The proteins were separated on a 12% polyacrylamide gel (200 V, 100 mA) and electroblotted using a Hoeffer electroblotting apparatus (model TE 42). A monoclonal antibody against the polymerase α subunit (generously supplied by Dr. Scott Lesley, McArdle Laboratory) was used as a probe. Blocking and detection followed procedures described by Lesley et al. [12].

To test whether the polymerase subunits were the only proteins being expressed, a one-dimensional polyacrylamide gel was run at the end of the experiment. The protocols used were the same as those described for the Western blot procedure. After electrophoresis, the gel was dried and overlain with a sheet of Kodak X-OMAT™ film.

Field generation. Duplicate EMF exposure coils were housed in separate incubators (Warren-Sherer RL-8); each coil (40 turns of No. 16 enameled copper magnet wire) was wrapped around the outside of a 500 ml cylindrical water-jacketed container (Fig. 1; LG8012, Lab Glass, Vineland, NJ, USA). To insure that temperature inside the containers would not be a factor, a

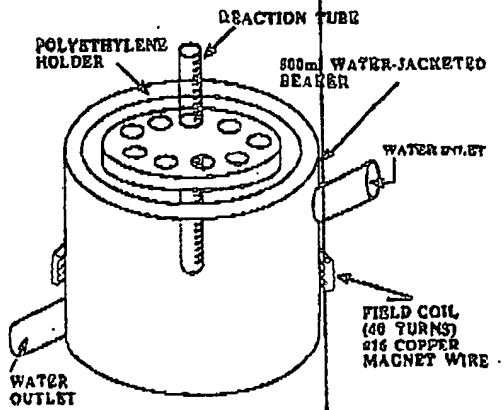

Fig. 1. A schematic drawing of the EMF exposure apparatus used for maintaining constant temperature in the cell-free expression system experiments.

common water bath maintained both units at 36.6°C. In addition, the equilibrium temperature was measured prior to each experiment in both the control and MF-containers with the fields on, using a Fluke (Model 52) thermocouple probe. In our initial exposures, a magnetic field of 72 Hz, 1.1 mT (rms) was generated by a Wavetek oscillator (Model 275) and a Techron amplifier (Model 7541). In later experiments the field intensity was reduced to 0.21 mT and 0.07 mT. The control configuration was similar to that of the exposure system, except that the coils were connected to a 'dummy' amplifier output impedance located outside the incubator. The conductivity of the final cell-free reaction mixture was measured to be 1.7 S/m. The 50-μl sample formed an 8 mm diameter pool in the bottom of the polystyrene test tube. The peak induced electric field at the edge of the sample at 1.1 mT was calculated to be 0.9 mV/m, corresponding to a current density of $J = 1.6$ mA/m^2. Total harmonic distortion was measured to be less than 0.1% for all harmonics up to approx. the 3 kHz cutoff of the gaussmeter (F.W. Bell, Model 640). To insure that we did not have undetected incubator effects, the control and experimental incubators were switched midway through the experiments described in this paper.

Statistical analyses. A nested analysis of variance was performed to compare the effects of the 1.1 mT, 72 Hz magnetic field on the amount of [^{35}S]methionine incorporated during protein synthesis of the α, β,β' or mixture in the cell-free expression system (This experimental design is also referred to as a partially-replicated design or split-plot design and designated A × B × C(B) in statistics texts [13]). A nested analysis is required because data for only a single plasmid were taken on a given day. The dependent variable for this analysis is the dpm of [^{35}S]methionine incorporated in proteins synthesized during the 60 min the transcription/translation mix was exposed to the field. Control samples were treated in an identical manner, except no field was applied. The effect of day (due largely to differences in specific activity of labelled methionine) was controlled for by explicitly including day as a variable. A logarithmic transformation of the data was performed to assure uniformity of variance and to eliminate interaction between independent variables in the analysis of variance.

To study the effects of different field levels and exposure times, multiple regression analysis was used to fit an equation to the data. Both linear and exponential models were tried. In this set of experiments there are too many variable levels to permit an explicit analysis of the (uninteresting) variable of day, so this variation is controlled for by performing a ratio of the average of counts from the four experimental tubes to

TABLE I

Analysis of variance on the effect of a 1.1 mT, 72 Hz magnetic field applied for 60 min on expression of RNA polymerase as a function of plasmid type, with day of experiment nested within the plasmid type

Plasmid type 'holo' refers to a 50:50 mixture of plasmids containing α and β,β' RNA polymerases. The dependent variable is the logarithm of the dpm from [^{35}S]methionine incorporated into proteins synthesized during the 60-min experimental period.

Sums of squares

	df	Sum of Sq	Mean Sq	*F*-value	*P*-value	Error term
Field	1	0.43	0.43	8.42	0.0337	field × day (plasmid)
Plasmid	2	0.82	0.41	0.14	0.8734	day (plasmid)
Day (plasmid)	5	14.73	2.95	341.80	0.0001	Residual
Field × plasmid	2	0.03	0.01	0.26	0.7784	field × day (plasmid)
Field × day (plasmid)	5	0.25	0.05	5.90	0.0003	Residual
Residual	42	0.36	0.01			

	Control			Exposed		
	n	Mean	S.E.	*n*	Mean	S.E.
Day 1, holo	4	5.94	0.04	4	6.04	0.01
Day 2, holo	4	4.50	0.05	4	5.01	0.08
Day 3, holo	2	5.57	0.03	3	5.69	0.02
Day 4, alpha	2	4.83	0.01	3	5.01	0.02
Day 5, alpha	4	5.80	0.04	4	5.88	0.01
Day 6, beta	4	4.61	0.07	4	4.91	0.04
Day 7, beta	4	5.82	0.08	4	5.94	0.02
Day 8, beta	4	4.90	0.04	4	4.97	0.06

the average of counts from the four control tubes. This conservative approach sacrifices some of the degrees of freedom (and thus power of the analysis) for simplicity.

Results and Discussion

The results of an analysis of variance of plasmids exposed to 1.1 mT, 72 Hz magnetic field are shown in Table I. We conclude that the magnetic field produces a significant and identical effect on two plasmids containing either α or β,β' subunits of RNA polymerase, or a mixture of the two plasmids.

The effect of the field is significant at $P = 0.03$. For this set of experiments the effect of day is controlled for by explicitly including day as a variable. An analysis of field effects on individual plasmid types shows that the field affects both plasmids and the mixture of plasmids in the same way (data not shown). These comparisons are made by forming 'contrasts' or different linear combinations of means in the table and performing an analysis of variance on the subset (Statistics texts also refer to contrasts as comparisons among several means [14]). The expected strong effect of day is clear from the large F-value in Table I for this variable: The significant interaction between field and day variables indicates the magnitude of the field effect differs from day to day, but an insignificant field × plasmid interaction shows that it does not depend upon which plasmid is being studied.

Duplicate Western blots were done to analyze the products of the cell-free expression system employing the α subunit of RNA polymerase (see Fig. 2A). Although one cannot reliably quantify Western blots, it is noteworthy that the data qualitatively show enhanced levels of the protein expressed under field exposure, relative to non-exposed controls, in agreement with the radioisotope data. To determine if other radioactive products might be contributing substantially to our counting data, a one-dimensional polyacrylamide gel was run on the products of the expression system. An autoradiogram shown in Fig. 2B shows the products of a cell-free expression system containing the α and β polymerase plasmids; radiolabelled proteins were not detected at either the origin or bottom of the gels. Based on these data we conclude that only the primary gene products are being expressed at a significant level in our system and that these represent the principal source of the counts obtained from the filters.

The effect of length of exposure and field intensity were examined using a 50:50 mixture of α and β,β' plasmids in cell-free transcription/translation systems exposed to fields of 0.07–1.1 mT for 5–45 min; the raw data are provided in Table II. A multiple regression

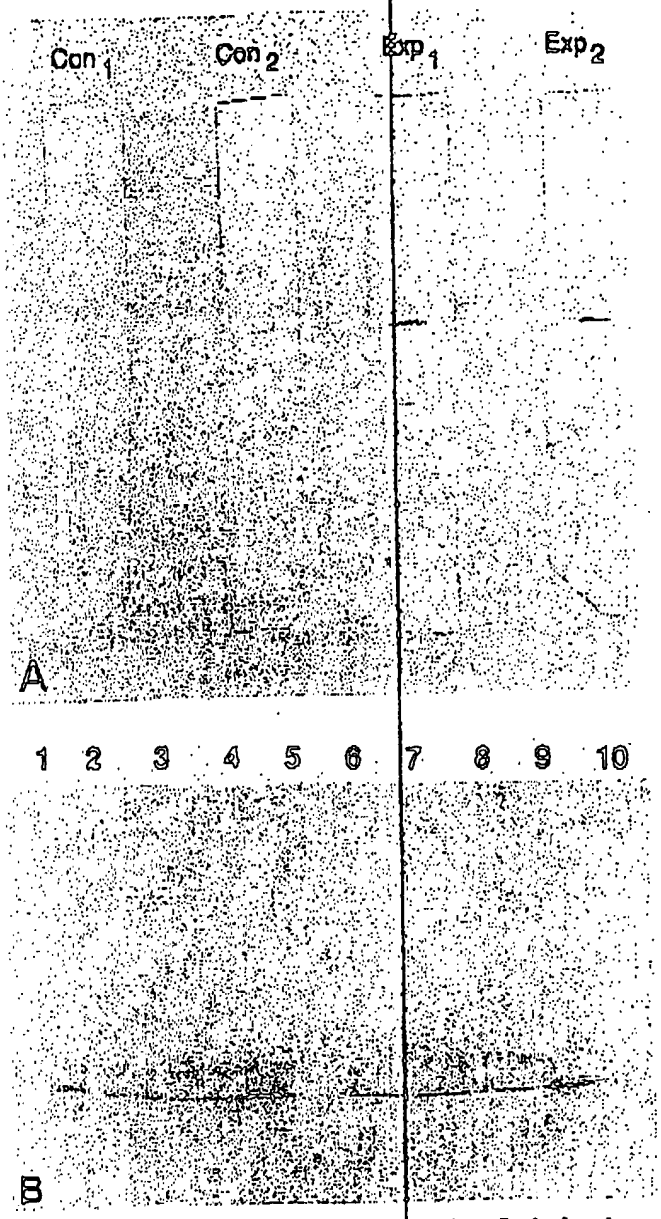

Fig. 2. (A), Western blot using a monoclonal antibody for the α subunit of DNA dependent RNA polymerase. Con 1 and 2 and Exp 1 and 2, respectively, represent samples from two controls and two MF-exposed samples obtained from a cell-free expression system. (B), A polyacrylamide gel of the products of an S-30 cell-free expression system following 45 min exposure to a 72 Hz, 1.1 mT electromagnetic field. Lanes 1–4 represent four samples of the nonexposed control products and lanes 6–9 represent four samples of the MF-exposed mixtures. The expression plasmids used in the experiment contained both the α (top band) and the β,β' (lower band) subunits of RNA polymerase. Lanes 5 and 10 represent the non-exposed and EMF-exposed negative controls.

analysis of these data yields an equation that best fits the data of

$$e/c = 0.96 \times (\mathrm{fi})^{0.18} \times (\mathrm{et})^{0.19} \quad (1)$$

where e/c is the ratio of dpm of [^{35}S]methionine incorporated during protein synthesis for the plasmid

Although these data show enhanced synthesis of a protein in a cell-free system, they do not directly address the question of whether the increase occurs as a result of enhanced transcription, translation, or both. Some insight into this question can be gotten from the experiments of Goodman et al. [16] who examined in-vivo transcription and translation in a variety of cell types. Their data show that exposure to weak MF results in an enhancement of transcription. Data from this lab (not shown) support the conclusion that weak field exposure increases RNA levels; whether this is due to enhanced synthesis or decreased degradation of RNA remains an open question.

Altered enzyme activity has also been reported in vitro when Na^+/K^+-ATPase was exposed to a constant electric field [17]. These experiments lend additional support to the notion that electromagnetic fields can directly alter physiological processes in the absence of an intact membrane.

In summary, we report that exposing a cell-free expression system to sinusoidal magnetic fields of 72 Hz, 0.07–1.1 mT for periods ranging from 5 to 60 min results in elevated levels of gene expression. Longer times are required to produce an effect at weaker fields. Of added significance is the fact that the increase occurred in the absence of an intact cell membrane. It is not immediately evident whether the increased levels of protein observed are a result of alterations in transcription, translation, or both. Alternatively, the stability of the mRNA might also be affected by field exposure. These data are in agreement with 2-D polyacrylamide gel experiments using intact *E. coli* in which the α subunit of RNA polymerase was observed to increase [7]. The use of a cell-free system provides a controlled environment to address the question of where and how weak fields interact with biological systems. However, even though this system offers a new model system for dissecting the mechanism of interaction between EMFs, additional in-vivo experiments will be required to establish the physiological relevance of these findings.

Acknowledgements

This work was supported by a grant from the Office of Naval Research. We thank Drs. Richard Burgess and Scott Lesley, McArdle Laboratory for Cancer Research for the technical assistance with the cell-free system and the generous gift of the α polymerase monoclonal antibodies. We also thank Dr. Henry Aldrich, University of Florida (Gainesville, FL, USA) for the electron microscopy and S. Beyerlein for excellent technical assistance.

References

1 Savitz, D.A., Wachtel, H, Barnes, F., John, E.M. and Tvrdik, J.G. (1988) Am. J. Epidemiol. 128, 21–38.
2 Shore, R.E. (1988) Cancer 15, 1767–1754.
3 Brown, H.D. and Chattopadhyay, S.K. (1988) Cancer Biochem. Biophys. 9, 295–342.
4 National Academy of Science USA (1986) Nonthermal effects of nonionizing radiation. Final report advisory committee on nonthermal effects of nonionizing radiation, Board on Radiation Effects Research, National Research Council.
5 Blank, M. and Findl, E. (eds.) (1987) Mechanistic Approaches to Interactions of Electric and Electromagnetic Fields with Living Systems, Plenum Press, New York.
6 Adey, W.R. (1988) in Charge and Field Effects in Biosystems, Vol. 2 (Allen, M.J., Cleary, S.F. and Hawkridge, F.M., eds.), pp. 263–290, Plenum Press, New York.
7 Goodman, E.M., Greenebaum, B. and Marron, M.T. (1993) Bioelectromagnetics 14, in press.
8 Zubay, G. (1973) Genetics 26, 267–287.
9 Jovanovich, S.B., Record, M.T. and Burgess, R.R. (1989) J. Biol. Chem. 264, 7821–7825.
10 Bedwell, D.M. and Nomura, M. (1986) Mol. Gen. Genet. 204, 17–23.
11 Phillips, T. (1988) DNA Protein Eng. Tech. 1, 5–9.
12 Lesley, S.A., Brow, M.A. and Burgess, R.R. (1991) J. Biol. Chem. 266, 2632–2638.
13 Lindman, H.R. (1974) in Analysis of Variance in Complex Experimental Designs, p. 173., W.H. Freeman, San Francisco.
14 Winer, B.J. (1962) in Statistical Principles in Experimental Design, p. 209, McGraw-Hill, New York.
15 Liestol, K. and Benestad, H.B. (1986) Exp. Hematol. 14, 187–191.
16 Goodman, R., Wei, L.-X., Xu, J.-C. and Henderson, A. (1989) Biochem. Biophys. Acta 1009, 216–220.
17 Blank, M. (1992) FASEB J. 6, 2434–2438.